Combination of guanine arabinoside and Bcl-2 inhibitor YC137 overcome the cytarabine resistance in HL-60 leukemia cell line.

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Abstract

Cytarabine (ara-C) is the key agent for treating acute myeloid leukemia. After being transported into leukemic cells, ara-C is phosphorylated, by several enzymes including deoxycytidine kinase (dCK), to ara-C triphosphate (ara-CTP), an active metabolite, and then incorporated into DNA, thereby inhibiting DNA synthesis. Therefore, the cytotoxicity of ara-C depends on the production of ara-CTP and the induction of apoptosis. Here, we established a new ara-C-resistant acute myeloid leukemia cell line (HL-60/ara-C60) with dual resistance characteristics of the anti-antimetabolic character of decreased ara-CTP production and an increase in the antiapoptotic factors Bcl-2 and Bcl-XL. We further attempted to overcome resistance by augmenting ara-CTP production and stimulating apoptosis. A relatively new nucleoside analog, 9-β-D-arabinofuranosylguanine (ara-G), and the small molecule apoptosis inhibitor YC137 were used for this purpose. The established subclone HL-60/ara-C60 was 60-fold more ara-C-resistant than the parental HL-60 cells. HL-60/ara-C60 cells exhibited low capacity for dCK protein expression, which resulted in decreased ara-CTP production. HL-60/ara-C60 cells were also refractory to ara-C-induced apoptosis due to overexpression of the antiapoptotic proteins Bcl-2 and Bcl-XL. Combination treatment of ara-C with ara-G augmented the dCK protein level, thereby increasing ara-CTP production and subsequent cytotoxicity. Moreover, the combination of ara-C with the Bcl-2 antagonist YC137 produced greater apoptosis than ara-C alone. Though the combination of ara-C with two drugs could only partially overcome ara-C

resistance in ara-C-resistant cells, the three-drug combination of ara-C, ara-G and YC137 substantially overcame resistance. These findings suggest possible combination strategies for overcoming ara-C resistance by augmenting ara-CTP production and reversing refractoriness against the induction of apoptosis in ara-C resistant leukemic cells.

Introduction

Cytarabine (1-β-D-arabinofuranosylcytosine; ara-C), a pyrimidine nucleoside analog, is key in the treatment of acute myeloid leukemia (AML).[1-3] Standard induction therapy, which consists of conventional doses of ara-C for 7 days plus anthracycline for 3 days, provides remission rates of over 70% for AML.[1-5] Nevertheless, only 40% of patients are long-term survivors and most relapse with the development of drug resistance. Thus, overcoming ara-C resistance is essential to improve clinical outcomes.[1-6]

Ara-C is transported into leukemic cells by membrane transporters including the human equilibrative nucleoside transporter 1 (hENT1).[7] Inside the cell, ara-C is phosphorylated to ara-C 5′-monophoshate by the rate-limiting enzyme deoxycytidine kinase (dCK) and subsequently to ara-C 5′-triphosphate (ara-CTP), an active metabolite of ara-C. Ara-CTP is then incorporated into DNA strands in the S phase of the cell cycle, resulting in the inhibition of DNA synthesis and the consequent induction of apoptosis.[1,8-11]

We have extensively investigated the mechanisms of resistance to ara-C to improve

therapeutic efficacy.[12-18] Because the toxicity of ara-C depends on its cellular activation to ara-CTP and the induction of apoptosis, cellular factors that can regulate ara-C activation and the apoptotic pathway may be critical in ara-C resistance. Thus, we hypothesized that strategies that could enhance ara-CTP production and stimulate apoptosis would augment ara-C's cytotoxicity and overcome resistance to it in leukemic cells.

Here, we report that we have established a unique new ara-C-resistant AML cell line with coexpression of anti-antimetabolic and antiapoptotic factors. We further attempted to overcome ara-C resistance by augmenting ara-CTP production and stimulating apoptosis. A relatively new nucleoside analog, 9-β-D-arabinofuranosylguanine (ara-G, the metabolite of nelarabine), and the small molecule apoptosis inhibitor YC137 were used for this purpose. Ara-G is a purine nucleoside analog that is similar to ara-C but that has a slightly different activation pathway. [19-22] YC137 disrupts the function of the antiapoptotic molecule Bcl-2, inducing cytochrome c release from mitochondria and activating caspase-9. YC137 has been shown to induce apoptosis of hematopoietic progenitor cells overexpressing Bcl-2.[23,24]

Materials and Methods

Chemicals and Reagents.

Ara-C was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ara-G was purchased from RI Chemical. Inc (Orange, CA, USA). YC137 was purchased from Calbiochem (Darmstadt,

Germany). All other chemicals were of analytical grade.

Development of an ara-C-resistant leukemic cell line.

Human leukemia HL-60 cells were cultured in RPMI1640 media supplemented with 10% heat-inactivated fetal bovine serum at 37˚C in humidified air containing 5% carbon dioxide. To develop an ara-C-resistant HL-60 variant, parental HL-60 cells were cultured in media containing ara-C. The cultures were observed daily and allowed to grow. Drug concentrations were gradually increased on subsequent passages, and an ara-C-resistant subclone (HL-60/ara-C60) isolated using the limiting-dilution method.

Growth inhibition assay.

HL-60 cells and HL-60/ara-C60 cells (1×10^5/ml) were incubated for 72 h with different concentrations of anticancer agents. The growth-inhibitory effects were evaluated using either the trypan blue dye exclusion assay or the sodium 3′-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay, according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA) with slight modifications.[18]

Determination of intracellular production of ara-CTP.

The acid-soluble fraction (nucleotide pool) was extracted from HL-60 cells or HL-60/ara-C60

cells (1×10^6/ml, 10 ml), treated or untreated. HPLC was then used to determine intracellular ara-CTP as described in previous studies.[12,15]

Nucleoside transport capacity.

To evaluate the capacity of membrane nucleoside transporters, ara-C uptake was quantified using the method of Wiley et al with slight modifications.[7,16] Non-facilitated drug uptake was determined in the presence of 3 μM nitrobenzylthioinosine (Sigma), which interferes with the function of membrane nucleoside transporters. The capacity of the transporter was determined as the difference between drug uptake in the absence and presence of nitrobenzylthioinosine.

Determination of dCK protein expression using Western blotting.

Protein expression of dCK was determined using Western blot analysis as described previously.[18] Mouse monoclonal anti-dCK, which was developed in the Department of Pediatrics, Mie University School of Medicine[25] and rabbit polyclonal anti-Bad, rabbit polyclonal anti-Bcl-2, rabbit polyclonal anti-Bcl-XL antibodies (Cell Signaling Technology, Beverly, MA, USA) and anti-actin antibody (Sigma-Aldrich) were used as the primary antibodies. Anti-mouse IgG antibody and anti-rabbit IgG-horseradish peroxidase-conjugated antibody (Amersham Biosciences, UK) were used as the secondary antibody. The band density was measured using an imaging densitometer.

Determination of apoptotic cell death using nuclear staining with Hoechst 33342 and flow cytometry.

Treated or untreated cells (1×10^5/ml) were incubated with 20 μg/ml Hoechst 33342 at room temperature for 15 min. The samples were observed by fluorescence microscopy, and cells with apoptotic morphology were determined by counting 100 cells per treatment and were also analyed using an Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Indianapolis, IN, USA). Flow cytometory was analyed with the use of FACSCanto II (BD Bioscience, NJ, USA).

Statistical analyses.

All statistical analyses were performed using Microsoft Excel 2007 software (Microsoft, Redmond, WA, USA). All graphs, curves and columns were generated using GraphPad Prism software (version 5.0) (GraphPad Software, Inc. San Diego, CA, USA). Values of $P \leq 0.05$ were considered statistically significant.

Results

Establishment of ara-C-resistant leukemic cells.

The XTT assay demonstrated that HL-60/ara-C60 cells were about 60-fold more resistant to ara-C than HL-60 cells (Table 1). HL-60/ara-C60 cells exhibited cross resistance to the similar nucleoside analogs gemcitabine (dFdC) and cladribine (2CdA) (Table 1). Thus, the

ara-C-resistant subclone (HL-60/ara-C60) was successfully established.

Intracellular ara-CTP production and ara-C-related factors.

Intracellular ara-CTP is a surrogate marker of ara-C-induced cytotoxicity, so the production of ara-CTP was evaluated in both cell lines. When cells were incubated for 6 h with different concentrations of ara-C, the production of ara-CTP increased in a concentration-dependent manner (Fig. 1A). However, the ara-CTP level was significantly lower in HL-60/ara-C60 cells than HL-60 cells, suggesting that ara-CTP is critical to the sensitivity of cells to ara-C.

For successful production of ara-CTP, ara-C must be transported into cells and phosphorylated to the ara-C nucleotide. When cells were pulsed with ara-C, the drug was rapidly incorporated in both cell lines (Fig. 1B). However, the analog uptake was invisibly lower in HL-60/ara-C60 cells than HL-60 cells. Moreover, HL-60/ara-C60 cells exhibited lower expression of the rate-limiting enzyme dCK than HL-60 cells (Fig. 1C). Therefore, these results suggest that the development of ara-C resistance in HL-60/ara-C60 cells was in part attributable to altered ara-C-related factors that resulted in the cells' low capability for ara-CTP production.

Sensitization of the ara-C-resistant variant by ara-G.

One strategy that increases the intracellular ara-CTP concentration is pre-treatment with a purine nucleoside analog such as fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine-5′-monophosphate, F-ara-AMP). [26,27] Theoretically, the intracellular active metabolite F-ara-A triphosphate inhibits ribonucleotide reductase, thereby

reducing the dCTP pool. This stimulates dCK activity, which then enhances the production of ara-CTP. Here, a relatively new purine nucleoside analog, ara-G, and conventional F-ara-A were similarly evaluated. Combination of a minimally toxic concentration (10 μM) of either ara-G or F-ara-A with 10 μM ara-C similarly enhanced ara-CTP production in both cell lines compared with treatment with ara-C alone (Fig. 2A and B). The combination effect was more potent for ara-G than F-ara-A in HL-60/ara-C60 cells (Fig. 2B). This increase in ara-CTP production is in accordance with the augmentation of dCK protein expression by the addition of ara-G to ara-C (Fig. 1C). Moreover, we demonstrated small-scale production of ara-GTP in both HL-60 and HL-60/ara-C60 cells on treatment with ara-G (Fig. 2C). The combination also enhanced ara-C's cytotoxicity in both cell lines (Fig. 2D). Thus, these results suggest that ara-G augments dCK expression, thereby increasing ara-CTP production and as a result enhancing ara-C cytotoxicity .

Expression of Bcl-2-family proteins in leukemic cells.

Many anticancer agents exert their cytotoxicity by inducing apoptotic cell death.[28,29] The Bcl-2 family of proteins contains key regulators of the mitochondrial pathway of apoptosis. Of these, Bcl-2 has been found to be overexpressed in many cancer cells, including B-cell-derived lymphomas. Western blot analysis demonstrated increased protein levels of antiapoptotic Bcl-2 and Bcl-XL in HL-60/ara-C60 cells compared with HL-60 cells (Fig. 3). Thus, inhibition of apoptotic function was suggested to be involved in the mechanisms of resistance to ara-C in HL-60/ara-C60 cells.

Sensitization of ara-C-resistant cells by Bcl-2 inhibitor.

Antiapoptotic Bcl-2 might represent a target for the treatment of cancers, typically those in which Bcl-2 is overexpressed. The Bcl-2 inhibitor YC137 was designed to inhibit the binding of the proapoptotic Bid BH3 peptide to Bcl-2, thus disrupting an interaction essential for antiapoptotic activity.[24] When ara-C-resistant HL-60/ara-C60 cells were treated with ara-C plus YC137, the induction of apoptosis was more prominent than in cells treated with ara-C alone (Fig. 4B and E). The combination also induced apoptosis in ara-C-sensitive HL-60 cells, and the enhancement was apparent (Fig. 4A and D). The addition of YC137 augmented the growth-inhibition effects of nucleoside analogs including ara-C, dFdC and 2CdA on HL-60/ara-C60 cells (Fig. 4C, Table 2). These results suggest inhibition of Bcl-2 as a possible strategy to overcome the resistance of leukemia cells to ara-C.

Sensitization of ara-C-resistant cells by the combination of ara-G and Bcl-2 inhibitor.

We examined the effect of the combination of ara-C with ara-G and Bcl-2 inhibitor, respectively, in HL-60/ara-C60 cells. However, the effect on ara-C resistance was poor. Therefore, we examined the effect of the combination of ara-G, YC137 and ara-C in HL-60/ara-C60 cells and found it to almost fully overcome ara-C resistance. In order to examine whether this phenomenon is specific to the HL-60/ara-C60 cell line, we observed the combination effect of the three drugs in HL-60/AD cell lines that we had previously established[13] and showed 34-fold resistance to ara-C and also expressed Bcl-2. Thus, ara-C resistance can be almost fully

overcome even in the HL-60/AD cell line (Table 3).

Discussion

Ara-C is one of the most effective chemotherapeutic agents used in the treatment of AML. However, the resistance of leukemic cells to ara-C remains a major drawback. We and others have extensively investigated the mechanisms of ara-C resistance *in vitro* and *in vivo.*[12-18] Deficient dCK activity, decreased nucleoside transporter content, decreased DNA polymerase sensitivity, overexpression of the cytidine deaminase gene, and enhanced cN-II activity have been reported to be associated with ara-C resistance *in vitro*.[30-32] Clinically, low hENT1 transcript level, increased cN-II expression, decreased dCK expression, and increased cN-II/dCK expression ratio were associated with poor therapeutic outcomes of ara-C-based chemotherapy.[33-35] Moreover, resistance to ara-C might develop due to the antiapoptotic nature of cancer cells including the X-linked inhibitor of apoptosis protein (XIAP) and Bcl-2.[36,37] Thus, the development of cellular resistance to ara-C is mainly a result of decreased capability to produce intracellular ara-CTP and inhibition of apoptosis.

Here, we have demonstrated a reduced capability to yield ara-CTP (Fig. 1) and resistance to the induction of apoptosis (Fig. 3) in a newly established ara-C-resistant leukemic cell line (HL-60/ara-C60). In this resistant cell line, reduced ara-CTP production was attributable to a reduction in dCK protein (Fig. 1C) while its antiapoptotic nature was due to overexpression of Bcl-2 and Bcl-XL (Fig. 3). The addition of the purine nucleoside analog ara-G to ara-C

augmented the dCK protein level (Fig. 1C), thereby increasing ara-CTP production (Fig. 2A and B) and cytotoxicity (Fig. 2D). The combination of the Bcl-2 antagonist YC137 with ara-C provided a greater amount of apoptosis than ara-C alone (Fig. 4). The contribution of Bcl-2 overexpression was also noted in the exertion of cytotoxic effects of dFdC and 2CdA (Fig. 4C).

Ara-G is a guanosine nucleoside analog that exerts specific cytotoxicity in T-lymphoblasts compared with myeloblasts and B-lymphoblasts.[20,21] No cytotoxic effect was shown in HL-60 and HL-60/ara-C60 cell lines (Table 1). Ara-GTP was produced in these cells (Fig. 2C), although at a much lower level than in T-cells. This is reported to be a result of reduced ara-GTP half-life in these cells when compared with T-cells.[38-40] Like ara-C, ara-G must be phosphorylated intracellularly to ara-G triphosphate for its cytotoxic effect as well as the augmentation of ara-CTP production. However, the activation pathway of ara-G is not identical to that of ara-C.[19-22] Ara-G is transported into the cell via both nitrobenzylthioinosine-sensitive and -insensitive equilibrative nucleoside transporters including hENT1 and concentrative nucleoside transporter 3.[19] Inside the cell, ara-G is phosphorylated to its monophosphate form by not only dCK but also the mitochondrial enzyme deoxyguanosine kinase. Therefore, ara-GTP has been suggested to be an active enhancer of ara-C cytotoxicity even in ara-C-resistant leukemic cells that decreased dCK activity (Fig. 2C). We have demonstrated that the addition of ara-G to ara-C increased dCK expression, ara-CTP production and subsequent cytotoxicity in HL-60/ara-C60 cells (Table 1, Fig. 1 and 2). On the contrary, F-ara-A, which utilizes the activation pathway in common with ara-C, was less effective for enhancing ara-CTP production

in HL-60/ara-C60 cells (Fig. 2B). Thus, these results suggest that ara-G has potential; to overcome cellular resistance to ara-C.

Apoptosis is important for normal development, host defense, and suppression of oncogenesis, and faulty regulation of apoptosis has been implicated in cancer. The Bcl-2 family of proteins contains key regulators of the mitochondrial pathway of apoptosis and includes both antiapoptotic molecules such as Bcl-2 and Bcl-XL, and proapoptotic molecules such as Bax, Bak, Bid, and Bad.[23] Many types of cancer overexpress antiapoptotic Bcl-2 family members.[24] Here, Bcl-2 protein was overexpressed in HL-60/ara-C60 cells (Fig. 3), suggesting that the altered apoptotic function would contribute to the development of resistance to ara-C. Addition of the Bcl-2 inhibitor YC137 to ara-C induced more apoptosis in HL-60/ara-C60 cells than ara-C alone (Fig. 4). Apoptosis was also enhanced when YC137 was combined with other nucleoside analogs (dFdC, 2CdA), to which HL-60/ara-C60 cells showed cross resistance (Fig. 4). These results suggest that the antiapoptotic nature mediated by Bcl-2 overexpression plays a critical role in ara-C resistance in leukemic cells.

Moreover, synergistic effects were observed on the combination of ara-G with ara-C and YC137 with ara-C in both HL-60/ara-C60 and HL-60/AD cells, though both only partially overcame ara-C resistance (Table 3). Therefore, we examined the effect of ara-G, YC137 and ara-C in both ara-C-resistant cell lines. The three-drug combination almost full overcame ara-C resistance in both ara-C-resistant cell lines (Table 3). These results suggest that addition of two drugs of different nature could dramatically augment ara-C cytotoxicity in ara-C-resistant cells.

In conclusion, the development of drug resistance in cancer cells is usually multifactorial. Our findings suggest that reduced ara-CTP production and refractoriness of the induction of apoptosis may be major mechanisms of resistance to ara-C in leukemic cells. We may need to examine the involvement of both ara-C activation and antiapoptosis, and provide tailor-made treatment to reverse ara-C resistance. Moreover, optimum combination strategies should be developed based on our understanding of the mechanisms of resistance in ara-C-based chemotherapy for leukemia.

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Disclosure Statement

The authors have no conflict of interest.

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Figure legends

Figure. 1.

(A) Production of intracellular ara-CTP. Cells (1 x 10^6 cells/ml, 10 ml) were incubated for 4 h with 1, 2, 5, 10 μM ara-C. The acid-soluble fraction was extracted from each sample and applied to HPLC. O, HL60, ●; HL60/ara-C60. Each value represents the mean±SD of at least three independent experiments. *P ≤ 0.05 (B) Transport of ara-C into parental and ara-C-resistant cells. Membrane nucleoside transport of ara-C was assessed by pulsing the cells with 0.32 μM tritiated ara-C for 0, 20, 40, and 60 s, followed by quantification of cellular drug uptake by scintillation counting. Open bars, HL-60 cells; closed bars, HL-60/ara-C60 cells. Each value represents the mean±SD of at least three independent experiments. (C) dCK protein expression. The cells (1 x 10^6 cells/ml, 10 ml) were incubated with 10 μM ara-G for 2 h (or not), followed by washing in media and incubation with 10 μM ara-C for 3 h. dCK protein expression was determined using Western blot analysis.

Figure. 2.

(A, B) Enhancement of ara-CTP production by ara-G. Cells (1 x 10^6 cells/ml, 10 ml) were incubated with 10 μM F-ara-A or ara-G for 2 h (or not), followed by washing in media and incubation with 10 μM ara-C for 3 h. (C) Cells (1 x 10^6 cells/ml, 10 ml) were incubated with 10 μM ara-G for 4h. The intracellular ara-GTP was measured using HPLC as described in Materials and Methods (A, B, C). (D) Growth inhibition was determined using the trypan blue dye exclusion

assay after the cells had been treated with ara-C plus ara-G as described above. Open bars, without ara-G; closed bars, with ara-G. Each value represents the mean±SE of at least three independent experiments. *P ≤ 0.05.

Figure. 3.

Protein expression levels of Bad, Bcl-XL, Bcl-2 in HL-60 cells and HL-60/ara-C60 cells; Western blot analysis.

Figure. 4.

(A, B, D, E) Enhancement of apoptotic cell death by the combination of ara-C and the Bcl-2 inhibitor YC137. Cells were treated with 0.005 μM ara-C (A,D), 0.05 μM ara-C (B,E) or 2.5 μM YC137, or both in combination for 72 h. Induction of apoptosis was determined using nuclear staining with Hoechst 33342 (A,B) and was analyzed by flow cytometry (D,E). (C) Cells were incubated with a given nucleoside analog with or without co-incubation with 2.5 μM YC137 for 72 h. The IC_{50} was determined using the XTT assay. Ara-C, cytarabine; dFdC, gemcitabine, 2CdA, cladribine. Open bars, HL-60 cells; closed bars, HL-60/ara-C60 cells. Each value represents the mean±SE of at least three independent experiments. *P ≤ 0.05.

Table 1. Sensitivity of HL-60 cells and HL-60/ara-C60 cells to anticancer agents

	IC_{50} (μM)		
	HL-60	HL-60/ara-C60	RR
Ara-C	0.08±0.003	4.91±0.23	61
dFdC	0.004±0.0002	0.12±0.03	30
2CdA	0.07±0.006	17.1±2.82	244
Ara-G	>1000	>1000	-
Ara-C + Ara-G	0.1±0.08	0.23±0.05	2.3
CPT-11	0.008±0.0002	0.04±0.004	5

Cells were treated with each agent for 72 h, followed by the determination of IC_{50} using the XTT assay. RR, relative resistance calculated as the ratio of the IC_{50} of HL-60/ara-C60 cells relative to that of HL-60 cells. Ara-C, cytarabine; dFdC, gemcitabine; 2CdA, cladribine; CPT-11, irinotecan; ara-G, 9-β-D-arabinofuranosylguanine; ND, not determined due to insensitivity to ara-G. Values for IC_{50} are the means±S.D.

Table 2. YC137 augmented growth inhibition effects of nucleoside analogs on HL-60/ara-C60 cells

Drugs	IC_{50} (μM)
YC137	5.8±0.34
Ara-C	4.91±0.23
Ara-C + YC137	0.3±0.23
dFdC	0.12±0.03
dFdC + YC137	0.05±0.02
2CdA	17.1±2.82
2CdA + YC137	3.9±1.8

Cells were incubated with a given nucleoside analog with or without co-incubation with 2.5μM YC137 for 72 h. The IC_{50} was determined using the XTT assay. Ara-C, cytarabine; dFdC, gemcitabine; 2CdA, cladribine. Values for IC_{50} are the means±S.D.

Table 3. Ara-G and YC137 augmented growth inhibition effects of ara-C on ara-C-resistant cells

	IC_{50} (μM)		
	HL-60	HL-60/ara-C60 (RR)	HL-60/AD (RR)
Ara-C	0.08±0.003	4.91±0.23 (61)	2.7±0.59 (34)
Ara-C + Ara-G	0.1 ±0.08	0.23±0.05 (2.3)	0.4±0.14 (4)
Ara-C + YC137	0.04±0.003	0.3±0.23 (7.5)	0.17±0.19 (4.3)
Ara-C + Ara-G + YC137	0.05±0.001	0.1±0.06 (2)	0.07±0.01 (1.4)

Cells were incubated with a given nucleoside analog with or without co-incubation with 10μM ara-G or 2.5μM YC137 for 72 h. The IC_{50} was determined using the XTT assay. RR, relative resistance calculated as the ratio of the IC_{50} of HL-60/ara-C60 and HL-60/AD cells relative to that of HL-60 cells. Ara-C, cytarabine; Ara-G, 9-β-D-arabinofuranosylguanine. Values for IC_{50} are the means±S.D.